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ABSTRACT. A sensitive and specific radioimmunoassay for human α-lactalbumin, a milk protein, has been developed in order to examine the effect of prolactin on the human breast in normal and diseased states. Samples of milk from nursing mothers and from men and women with galactorrhea were found to contain milligram concentrations of this protein. In serum, 8 of 25 normal men and 18 of 44 normal women had detectable concentrations of α-lactalbumin. Significantly higher levels of α-lactalbumin were found in 17 of 19 women during pregnancy who were not actively lactating. All nursing mothers were found to have distinctly elevated serum α-lactalbumin concentrations. In a group of 17 female patients with phenothiazine induced prolactin elevations (mean 29.4 ng/ml), the mean serum α-lactalbumin of 17.3 ng/ml was significantly higher than in normal female volunteers. Patients with gynecomastia were not noted to have elevated α-lactalbumin.

In vitro, homogenates of normal breast and carcinoma tissue from the same individuals revealed that in 9 of 17 patients α-lactalbumin was present in higher concentrations in normal than in cancerous tissue. Overall, α-lactalbumin was found in 48.5% of homogenates and 41% of organ cultures of normal breast tissue from cancer patients. In contrast, it was present in only 19% of homogenates and 21% of cultures of carcinoma tissue, indicating that the cancer tissue may lose its ability to produce α-lactalbumin.

Differences in biologic behavior were found in some tumors. In 2 cases homogenates of breast cancer tissue had much higher concentrations of α-lactalbumin than the normal tissue, and in 3 of 33 tumors studied in organ culture prolactin increased α-lactalbumin output. (*J Clin Endocrinol Metab* 45: 1238, 1977)

ANIMAL studies show that prolactin stimulates milk protein synthesis (1,2) in addition to being necessary for mammary gland development (3) and possibly for stimulation of fatty acid synthesis (4). Prolactin has also been found to have important effects on the growth and maintenance of mammary cancer in rats (5,6). A major obstacle to determining the effects of prolactin on human breast in normal physiologic states and on the role of prolactin in human breast cancer has been lack of a suitable biochemical marker for the effects of prolactin. We have recently developed a radio-immunoassay for a human milk protein, α-lactalbumin (7), to evaluate the effects of prolactin in humans and in particular the role of prolactin in breast cancer.

Materials and Methods

α-Lactalbumin was isolated from whole human milk which was defatted and dialyzed against distilled water at 3 C. Casein was removed by acid precipitation as previously described (8). The lyophilized supernatant (2.2 g) or whey fraction, which contains the α-lactalbumin, was dissolved in 25 ml 0.005 M sodium phosphate at pH 8.2 and applied to a 2 × 50 cm DEAE cellulose column. The proteins were eluted at 72 ml/h by stepwise buffer changes at 0.01, 0.02, 0.05 and 0.10 M phosphate buffer at pH 8.2, and then by 0.1M phosphate buffer in 0.3M sodium chloride at pH 5.9. Most of the α-lactalbumin was found in peak II of the 0.05M buffer. After dialysis and lyophilization, the protein was further purified on a 2 × 57 cm Bio-gel P100 column in 0.025M sodium phosphate at pH 7 at 3 C. At an elution rate of 18 ml/h α-lactalbumin was found in a large peak following an initially smaller one. Approximately 1 mg of α-lactalbumin was

isolated per ml of skim milk used. The α-lactalbumin was found to migrate as a single band on disc gel electrophoresis at pH 9.6 (Fig. 1), and as a single band on an SDS gel. The amino acid composition of this preparation, done by Gordon (unpublished observations) was found to be virtually identical to previously published amino acid compositions of human α-lactalbumin (9,10).

Antisera to this pure α-lactalbumin were raised in New Zealand white rabbits by injecting 1 mg of protein in 1 ml of complete Freund's adjuvant at days 1 and 30. At 60 days, a booster was given and antibody titers were examined 1 week later. The antiserum used for these studies (#7-1141) was employed at concentrations of 1 to 375,000. Previously, we used an antiserum to whole human milk (7) with high titer anti-α-lactalbumin antibodies. To compare the 2 antisera, 58 separate serum samples with measurable α-lactalbumin were examined simultaneously in assays employing both antisera. There was excellent agreement between results obtained (Fig. 2).

Pure α-lactalbumin was used both for iodination with ^{125}I by the method of Hunter and Greenwood (11), and for standards. 10 standards ranging from 0 to 4 ng/ml of α-lactalbumin were employed. All samples were run in duplicate. Each assay tube contained a total incubation volume of 0.5 ml. Serum samples occupied no more than 1/20th of the final volume in order to minimize non-specific damage. The reaction was carried out for 5 days at 4 C. Bound α-lactalbumin was separated from free by a double antibody technique using sheep anti-rabbit gamma globulin. This reaction was carried out at room temperature for 24 h and then separated by centrifugation. Figure 3 is a representative standard curve. Sensitivity ranges between 0.02 ng/ml and 0.05 ng/ml.

The intra-assay precision was determined by taking the α-lactalbumin values of duplicate tubes and obtaining the difference between duplicates as a per cent of the mean. The mean of the differences between duplicates in representative assays was 5.6%. The inter-assay precision was determined by taking 6 separate pools of human serum with different concentrations of α-lactalbumin and measuring the content in different assays. The results expressed as the coefficient of variation can be found in Table 1. When a pool of serum without detectable α-lactalbumin was evaluated, α-lactalbumin was not detected in 18 separate assays, but was found at a concentration of 1.2 ng/ml in the 19th. The variation between assays was highest at the upper part of the immunoassay curve. Overall, the coefficient of variation ranged from 12.5% to 28.2%.

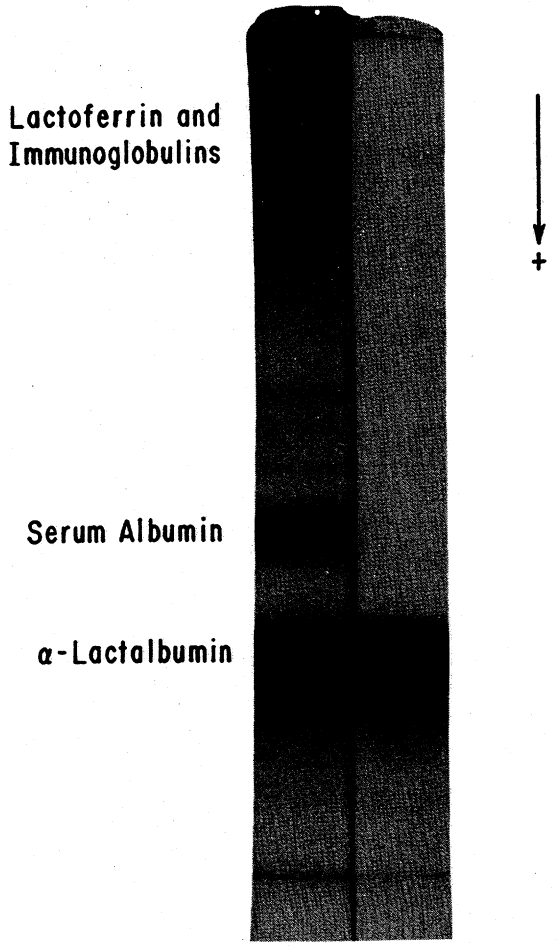

FIG. 1. Disc gel electrophoretic pattern of whey proteins (left) and α-lactalbumin (right) extracted from the milk of a single individual.

Specificity studies were carried out by determining if other proteins in high concentrations caused displacement of the standard curve. No α-lactalbumin was detected in human preparations of lysozyme (1 μg/ml), lactoferrin (1 mcg/ml), casein (1 μg/ml), milk serum albumin (1 mcg/ml), prolactin (0.7 μg/ml) or gamma globulin (1.4 μg/ml).

Tissues for homogenization were kept frozen until assayed. They were then thawed, weighed, and homogenized in 2 ml of 0.05M phosphate

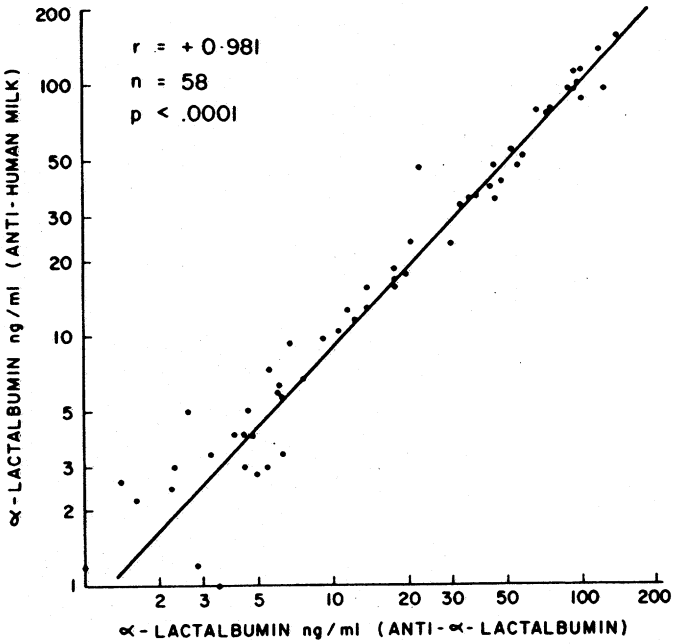

FIG. 2. Comparison of α-lactalbumin in 58 individual serum samples measured by radioimmunoassay using one antiserum raised to pure human α-lactalbumin and another to human milk. Each dot represents a single serum measured in both systems.

buffer with a Polytron homogenizer at a setting of 7.5 for two 15-sec bursts. After centrifugation, α-lactalbumin measurement was carried out on the supernatant. Results were expressed in pg/mg tissue.

Organ culture studies were carried out as previously described (7). In essence, fragments of breast tissue were placed on grids with lens paper in Falcon organ culture dishes and maintained in medium 199 with insulin (10 μg/ml) and hydrocortisone (20 μg/ml) at 37 C. in an atmosphere of 95% O_2–5% CO_2. α-Lactalbumin-free pooled human female serum in 20% concentrations was added to cultures, but serum-free cultures were examined as well.[1] Ovine

[1] Early in our studies one pool of serum contained α-lactalbumin (7.8 ng/ml). We have not, therefore, used results from experiments with this serum pool unless definite stimulation by added prolactin was observed.

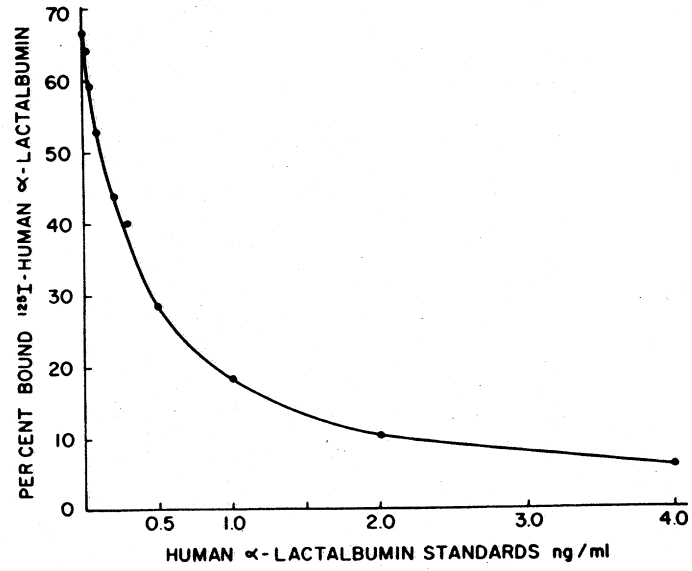

FIG. 3. Representative standard curve of α-lactalbumin radioimmunoassay.

prolactin was added in concentrations of 100 and 1000 ng/ml.[2]

Results

Half-time disappearance of α-lactalbumin from serum

To determine the disappearance of α-lactalbumin from blood, 1 mg of the human material was given by bolus iv injection to each of 3 male baboons. Serial blood samples were drawn before and after injection of the α-lactalbumin. No α-lactalbumin was detectable in 3 baseline samples from each animal. Peak serum levels of α-lactalbumin as measured by immuno-assay were found at 1 and 2 minutes after injection. The disappearance of α-lactalbumin in a representative animal is shown graphically in Fig. 4. In this animal the half-time was calculated as 9.1 min with a volume of distribution of 1320 ml. After an initial rapid decline in α-lactalbumin levels, the disappearance was expressed by a line as determined by the method of least squares for the 5 time periods between 5 and 60 min. Low concentrations of α-lactalbumin were detectable for 5 h after injection. Similar results were obtained in the other 2 animals in whom half-times were 10 and 10.2 min.

α-Lactalbumin in human milk

Milk samples from 7 nursing mothers and 5 women and 2 men with galactorrhea of varying etiologies were examined for α-lactalbumin content. The milk was first centrifuged to remove the creamy layer. Results which are found in Table 2 indicate that milligram concentrations of α-lactalbumin are contained in milk samples of both nursing mothers and galactorrhea patients. It should be pointed out that milk volumes were far greater in the nursing mothers than in the galactorrhea patients, in some of whom only microliter volumes were expressible at any one time.

TABLE 1. Precision of α-lactalbumin radioimmunoassay

Mean α-lactalbumin (ng/ml)	Number of assays	Coefficient of variation (%)
<1	19	*
3.7	18	28.2
6.8	19	14.1
9.2	14	23.1
23.2	8	20.9
104.4	17	12.5

* α-Lactalbumin was undetectable in 18 of 19 assays, and detectable in one at 1.2 ng/ml.

α-Lactalbumin in blood (Fig. 5)

Normal women and men. Serum samples from a group of ambulatory people without known medical or endocrine disorders were evaluated for α-lactalbumin content. Of 44 normal women ranging in age from 5 to 70

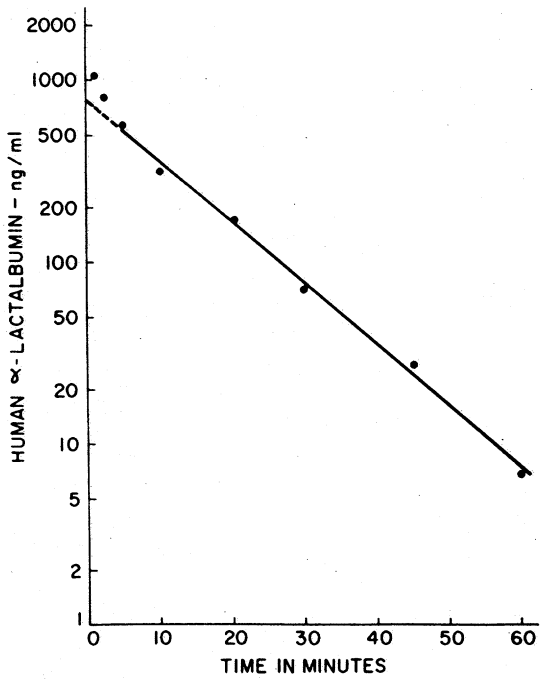

FIG. 4. Disappearance of human α-lactalbumin from serum of a male baboon injected with 1 mg of the pure material. Each point represents the measurement of α-lactalbumin by radioimmunoassay at a different time.

[2] Millipore filter sterilization resulted in ovine prolactin losses of approximately 33%. The final effective concentration of ovine prolactin in cultures was therefore 67 and 670 ng/ml.

TABLE 2. α-Lactalbumin in human milk

Subjects	No.	Mean α-lactalbumin (mg/ml)	Range (mg/ml)
Nursing mothers	7	2.07	0.5–5.6
Galactorrhea patients			
(Females)	5	2.82	1.5–3.8
(Males)	2	0.95	0.9–1.0

years, α-lactalbumin was found in 18. The mean concentration, assuming 0 for serum samples in which α-lactalbumin was not detected, was 2.18 ng/ml ± 3.6 (SD). α-Lactalbumin was found in 8 of 25 normal males ranging in age from 7 to 73, with a mean of 1.6 ng/ml ± 2.7.

Pregnancy. α-Lactalbumin was found in the blood of 17 of 19 pregnant women, the gestation periods ranging from 4 to 40 weeks. Mean α-lactalbumin for this group as a whole was 35.4 ng/ml, which was significantly greater than the α-lactalbumin among the normal non-pregnant women ($P < 0.001$). Serum prolactin ranged widely from 3.2 to 190 ng/ml with a mean of 59.7 ng/ml.

Nursing mothers. α-Lactalbumin in a group of nursing mothers has been previously reported (7) and this group of 18 is included as a point of reference. The mean α-lactalbumin of 149 ng/ml was higher than in any other group we have studied. No significant correlation between serum α-lactalbumin and prolactin was noted. Mean prolactin was 70.5 ng/ml.

Phenothiazine patients. A group of 17 female psychiatric patients, with diagnoses of schizophrenia, who were receiving large doses of phenothiazine-like drugs for periods ranging from 10 days to several years, were tested. The mean age of these patients was 29.7 years (range: 19–44). α-Lactalbumin was found in 14 of the 17 patients with a mean concentration of 17.3 ng/ml. Although α-lactalbumin levels in 8 patients overlapped with measurable values in normal individuals, the mean for the group was significantly higher than in the normal female controls ($P < 0.001$). Prolactin ranged from 11.5 to 64 ng/ml and the mean was 29.4 ng/ml.

Gynecomastia patients. α-Lactalbumin was measured in the serum of 21 patients with gynecomastia of varying etiologies. Detectable levels were found in 10. The mean for the group as a whole was 2.3 ng/ml,

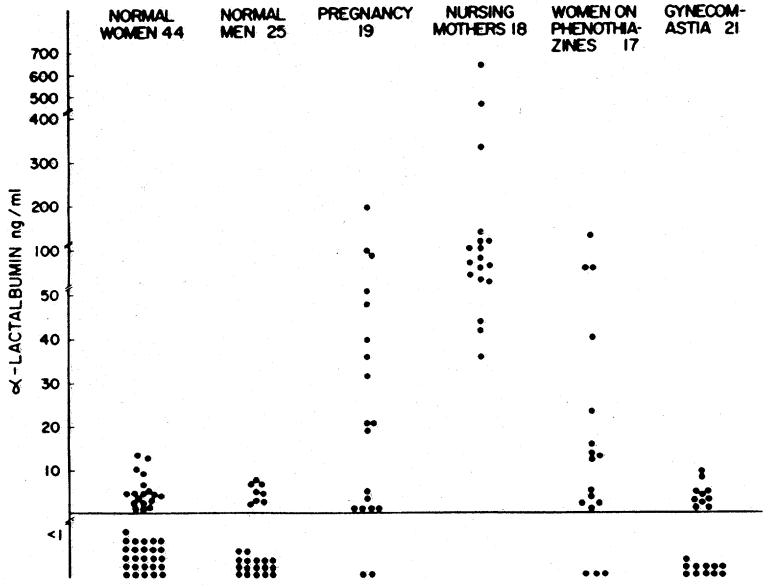

FIG. 5. Human α-lactalbumin measurements (ng/ml) in individual patients.

assuming 0 for unmeasurable values. There was no significant difference in α-lactalbumin measurements between these patients and the normal male volunteers. There was also no correlation between prolactin and α-lactalbumin levels, the mean prolactin being 10.2 ng/ml.

α-Lactalbumin in homogenates of breast cancer and normal breast tissue

In order to examine the relationship of α-lactalbumin found in normal breast and breast cancer tissue, samples of these tissues were obtained from the same breast from 17 patients with primary breast cancer. The normal tissue was obtained from sites as far away from the tumor as possible. Results of α-lactalbumin measurements are depicted in Fig. 6. In 8 of the 17 patients, α-lactalbumin was found in samples of normal breast tissue in concentrations ranging from 1.5 to 550 pg/mg of tissue. No detectable α-lactalbumin was found in breast carcinoma tissue from these same individuals. Another patient who was 38 years old and 8 weeks pregnant at the time of surgery for breast cancer had higher levels of α-lactalbumin in her normal breast tissue (950 pg/mg) than in the carcinoma (15.1 pg/mg). In contrast, 2 patients had higher concentrations of α-lactalbumin in their tumor tissue than in the normal. In one, α-lactalbumin was undetectable in the normal tissue but there was 150 pg/mg in the carcinoma. In the other, high levels (1300 pg/mg) were found in the normal tissue with much higher levels 22,000 pg/mg) in the cancer tissue. In 6 patients no detectable α-lactalbumin was noted in either cancer or normal tissue.

Overall, α-lactalbumin was found in a greater percentage (48.5%) of histologically

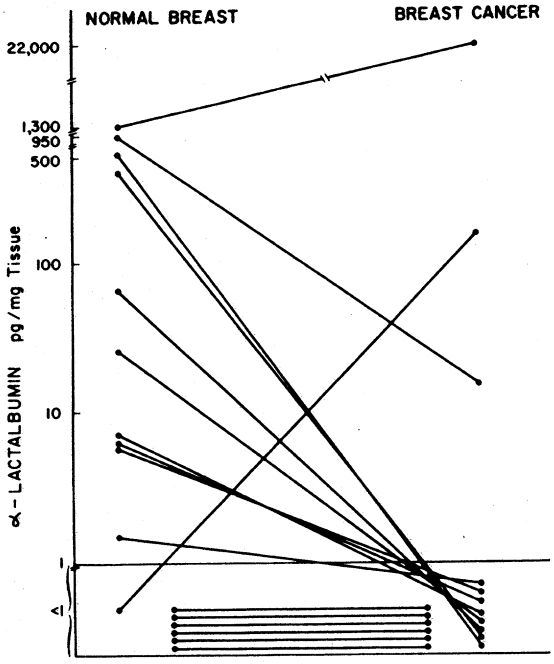

FIG. 6. α-Lactalbumin in homogenates of normal breast and breast cancer from the same patients. Solid lines connect α-lactalbumins in normal and cancer tissue from individual patients.

normal breast tissue from cancer patients than in the cancer tissue itself (19%) as seen in Table 3. The difference between the presence or absence of α-lactalbumin in normal and cancer tissue was highly significant ($P < 0.005$) as determined by the chi-square test. However, the range of α-lactalbumin when detectable was wide, and the difference between the mean of the measurable values was not statistically significant. There was no correlation noted between α-lactalbumin detectability and age. Of the patients in whom α-lactalbumin was measurable in the normal breast tissue, 10 of 14 were no longer menstruating. Of those

TABLE 3. α-Lactalbumin in homogenates of normal breast and breast cancer

Tissue	Number positive	Total number of samples	Per cent positive	Mean α-lactalbumin (pg/mg)	Range (pg/mg)
Normal breast	16	33	48.5%	3779	2.3–55,000
Breast cancer	12	63	19%	2383	2.9–22,000

who had no detectable α-lactalbumin in the normal tissue, 15 of 19 were not menstruating. The patient in whom α-lactalbumin was highest in the normal tissue (55,000 pg/mg) was not menstruating but was receiving high doses of thioridizine and imipramine prior to and at the time of surgery.

The histological appearance of the cancer tissue was not helpful in predicting which specimens would contain α-lactalbumin. In most cancers, although the architecture of the tissue was disordered, there were a greater number of epithelial cells than in the normal tissue. This was true even when α-lactalbumin was found in the normal but not the cancer. To illustrate this, histologic sections of normal and cancer tissue from a patient who had detectable α-lactalbumin in

FIG. 7A and 7B. Normal breast (7A) and carcinoma (7B) from a 61 year old patient with homogenate α-lactalbumins of 6.4 pg/mg and <1.0 pg/mg respectively.

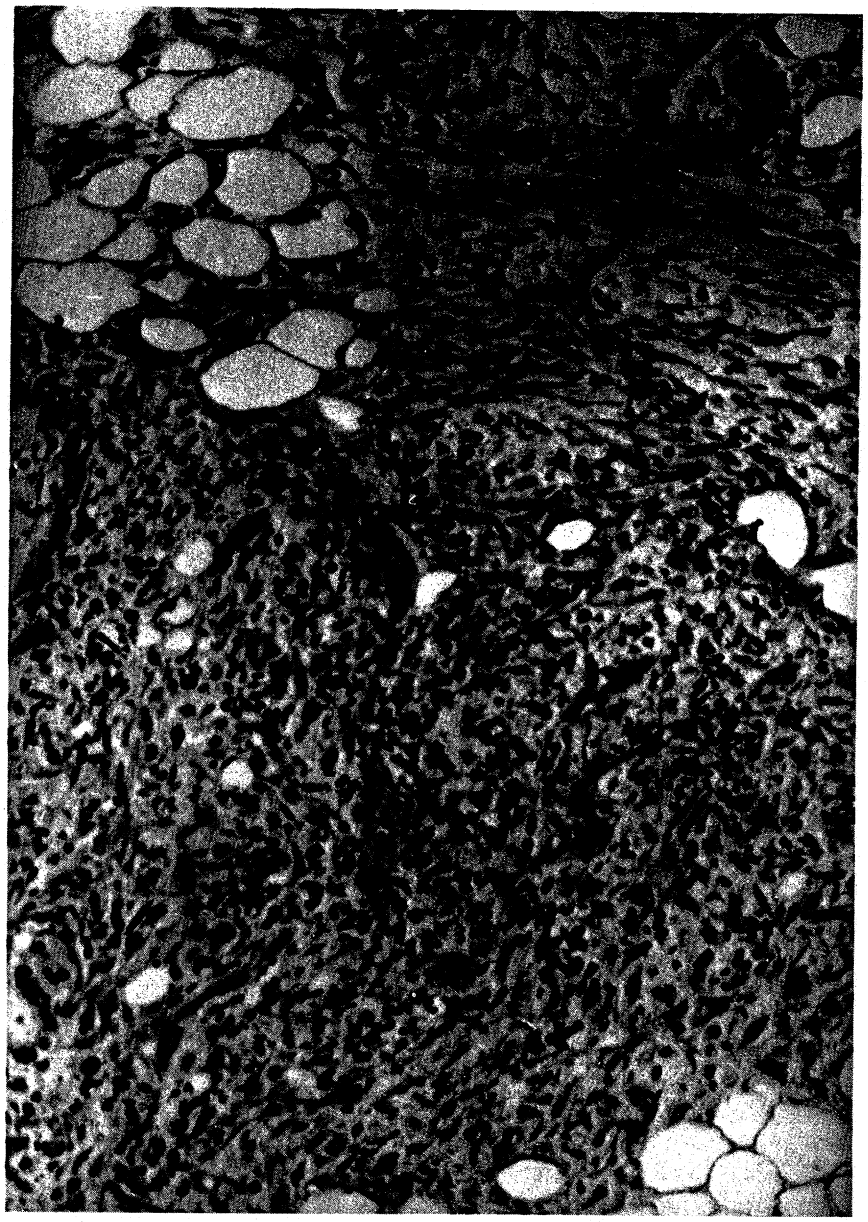

FIG. 7B. See legend to Fig. 7A.

the normal tissue but not in the carcinoma can be seen in Figs. 7A–B.

Organ Culture studies

To determine the effect of prolactin on breast cancer and normal breast tissue, fragments of tissue were maintained in organ culture for periods of 3 days with and without added prolactin. α-Lactalbumin was then measured in the medium bathing the tissue. Of 33 breast cancers examined in this way, α-lactalbumin was detected in the baseline medium of 7, and α-lactalbumin was found in the medium of 9 of 22 histologically normal breast tissue samples taken from patients with cancer (Table 4). When ovine prolactin was added to the medium α-lactalbumin output was increased in 3

failure of ergot drugs to lower growth hormone, which possesses a high degree of intrinsic lactogenic activity (32,33) results in insufficient inhibition of lactogenic activity in prolactin-sensitive tumors. Another reason for this discrepancy may be that patients with prolactin-sensitive tumors were not the ones selected for drug treatment. Also, a combination of these explanations may be applicable. A major obstacle to examination of the role of prolactin in human breast cancer has been lack of a satisfactory means of determining which tumors may be prolactin-sensitive. We have tentatively used the presence of α-lactalbumin in tissue as an indicator of prolactin sensitivity in human tumors, although such a relationship can be firmly established only by the demonstration of increased α-lactalbumin production after the addition of prolactin to organ cultures of breast tissue. The results presented here, which expand previously published data from our laboratory (7), indicate that 19% of primary breast tumors have detectable α-lactalbumin in tissue homogenates and a similar number (21%) of tumors are capable of secreting α-lactalbumin into organ culture medium. In 3 tumors in culture, prolactin added to the medium caused a further increase in α-lactalbumin secretion. Why some tumors in culture, or for that matter most normal tissues from these cancer patients, failed to respond to added prolactin is not entirely clear; cultured mammary tissues from baboons and monkeys respond readily to added prolactin (2) with significant increases in α-lactalbumin output. This difference between normal tissue in subhuman primates and humans might be explained by the fact that the normal human tissue came from patients generally outside the childbearing years, all but 2 being menopausal, and that prior hormonal *in vivo* stimulation of the breast tissue was inadequate compared to the animals referred to above, who were in the reproductive years. On the other hand, one must consider the possibility that there are species differences or that patients with carcinoma have abnormalities in histologically normal tissue. Our homogenate studies on α-lactalbumin are in accord with those of Monaco *et al.* (34) who found that 17% of breast cancer cytosols contained casein. Methods other than milk protein determination have been used to examine prolactin sensitivity. Studies on prolactin receptors have proven technically difficult because of low binding. One preliminary report found that binding of greater than 0.9% was significant and that degree of specific binding was found in approximately 20% of tumors (35). A higher number of tumors (32%) were found to be prolactin responsive by the less specific method of determining glucose-6-phosphate dehydrogenase activity as endpoint (36). If one assumes that the presence of α-lactalbumin or other milk proteins in tumors is indicative of prolactin sensitivity, then measurement of α-lactalbumin in biopsies of tumors or metastatic lesions may eventually provide a means of pre-selecting patients who might respond to prolactin-inhibiting drugs or hypophysectomy.

It is not surprising that α-lactalbumin was found in a greater number of specimens of histologically normal breast tissue from patients with breast cancer than in breast cancer tissue itself; milk production is the major function of normal breast tissue. Our findings in studies of normal and cancer tissue from the same breast show that in 8 patients α-lactalbumin was found in the normal tissue only. In another patient, who was 2 months pregnant at the time of surgery, α-lactalbumin was present in the normal tissue in concentrations 63-fold greater than in the cancer tissue and this patient's carcinoma tissue when maintained in culture did not produce detectable amounts of α-lactalbumin even when prolactin was added. These data suggest that in these patients the malignant breast tissue has lost its ability to produce α-lactalbumin in measurable concentrations. The abnormality in these carcinoma tissues may range from defects in the receptor to the final production of α-lactalbumin. That there are major differences in

the biological behavior of breast cancers is suggested by the fact that α-lactalbumin was found in much higher concentrations in carcinoma than normal tissue in 2 individuals, indicating that these tumors might be even more sensitive to the action of prolactin than the normal tissue or that overproduction of α-lactalbumin may be occurring in these tumors independent of prolactin. Our studies indicate that measurement of α-lactalbumin provides a biochemical marker for examining the effects of prolactin on mammary tissue and measurement of this milk protein may eventually prove valuable in helping to decide on treatment regimens in individual patients with breast cancer, once this hypothesis is clinically evaluated.

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